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NOTE

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Chemical Constituents of a Marine Fungus, *Arthrinium sacchari*

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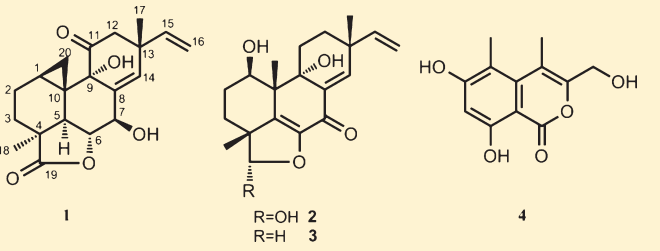
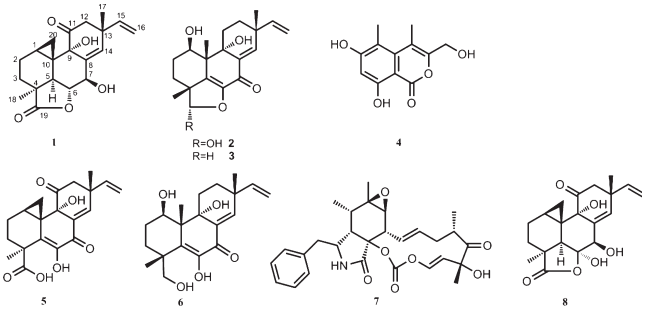
Supporting Information

ABSTRACT: Three new diterpenes, myrocin D (**1**), libertellenone E (**2**), and libertellenone F (**3**), and a new isocoumarin, decarboxyhydroxycitrinone (**4**), were isolated from the marine fungus *Arthrinium sacchari*, together with three known compounds (**5**−**7**). The structures of **1**−**4** were elucidated from spectroscopic data (NMR, MS, IR), and the absolute configurations of **1**−**3** were determined by X-ray diffraction analysis. The antiangiogenic activity of these compounds was evaluated by measuring their antiproliferation effects on human umbilical vein endothelial cells (HUVECs) and human umbilical artery endothelial cells (HUAECs). Compounds **4**−**7** showed inhibitory activity.



Marine fungi have yielded many new chemical compounds, with the highest number being isolated from fungi collected from sponges and algae. We previously reported a novel compound, phomactin (phomactin H), which was isolated from a marine-derived fungus found in brown algae.[1]

We previously described a screening assay using human umbilical vein endothelial cells (HUVECs) and human umbilical artery endothelial cells (HUAECs) for evaluating the ability of compounds to inhibit tumor angiogenesis. Several extracts of marine-derived fungi showed substantial inhibitory activity against HUVECs and HUAECs. In this paper, we describe the isolation and structural elucidation of three new diterpenes, myrocin D (**1**), libertellenone E (**2**), and libertellenone F (**3**), and a new isocoumarin, decarboxyhydroxycitrinone (**4**). In addition, isolated compounds (**1**−**7**) were evaluated for their ability to inhibit the proliferation of HUVECs and HUAECs. The seven metabolites isolated from the marine fungus *Arthrinium sacchari* (obtained from the surface of a sponge) inhibited the proliferation of HUVECs.



A. sacchari was grown on wheat medium, then extracted with $CHCl_3$ and EtOAc. The $CHCl_3$ extract was subjected to silica gel column chromatography (CC), HPLC, and Sephadex LH-20 CC. Two new compounds, myrocin D (**1**) and libertellenone F (**3**), and a known compound, cytochalasin E (**7**),[2] were obtained. The EtOAc extract was also subjected to silica gel CC, HPLC, and Sephadex LH-20 CC. Two new compounds, libertellenone E (**2**) and decarboxyhydroxycitrinone (**4**), and two known compounds, myrocin A (**5**)[3] and libertellenone C (**6**),[4] were obtained.

The molecular formula of compound **1** was determined to be $C_{20}H_{24}O_5$, based on HREIMS. The IR spectrum exhibited absorptions at 3400 cm^{-1} (hydroxy), 1740 cm^{-1} (γ-lactone), and 1715 cm^{-1} (ketone). The ^{13}C NMR spectrum (Table 1) showed one carbonyl carbon signal at C-11 (δ_C 210.0), one ester carbonyl carbon signal at C-19 (δ_C 184.2), and two methyl carbon signals at C-18 (δ_C 27.7) and C-17 (δ_C 28.0). Furthermore, the ^1H NMR spectrum (Table 1) showed four olefinic protons at H-14 (δ_H 6.25), H-15 (δ_H 5.81), H-16a (δ_H 4.98), and H-16b (δ_H 5.03) and a cyclopropyl group at H-20 (δ_H 0.57 and 0.10). The HMBC correlations from H-16 to C-13 and C-15, H-14 to C-9, C-12, C-13, and C-17, H-7 to C-6, C-8, and C-14, and H-5 to C-3, C-4, C-6, C-7, C-10, and C-18, and the $^1H-^1H$ COSY correlation from H-2 to H-3, suggested the presence of a pimarane-type diterpene. The $^1H-^1H$ COSY correlation from H-20 to H-1 indicated that the cyclopropyl group of C-20 was attached to C-1 and C-10, as did the HMBC correlations from

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Table 1. NMR Spectroscopic Data (400 MHz, CD_3OD, δ in ppm) for Myrocin D (1)

position	δ_C		δ_H (J in Hz)	HMBC
1	19.1	CH	2.06, m	
2α	20.7	CH_2	1.92, m	
2β			1.73, ddd (14.2, 6.1, 3.0)	
3α,β	24.0	CH_2	1.42, m	
4	40.2	qC		
5	41.5	CH	2.95, d (11.5)	3, 4, 6, 7, 10, 18
6	86.0	CH	3.85, dd (11.5, 9.5)	
7	75.2	CH	4.52, dd (9.5, 2.2)	6, 8, 14
8	140.5	qC		
9	76.8	qC		
10	28.5	qC		
11	210.0	qC		
12α	52.0	CH_2	2.69, d (13.9)	11, 13, 14, 15, 17
12β			2.33, d (13.9)	11, 13, 14, 15, 17
13	42.1	qC		
14	135.2	CH	6.25, d (2.2)	9, 12, 13, 17
15	145.4	CH	5.81, dd (17.3, 10.5)	13, 17
16a	113.1	CH_2	4.98, dd (10.5, 0.9)	13, 15
16b			5.03, dd (17.3, 0.9)	13, 15
17	28.0	CH_3	1.19, s	12, 13, 14, 15
18	27.7	CH_3	1.29, s	3, 5, 19
19	184.2	qC		
20α	9.6	CH_2	0.57, dd (5.7, 4.2)	2, 5, 10
20β			0.10, dd (8.7, 5.7)	2, 5, 9, 10

H-20 to C-2, C-5, C-9, and C-10. The spectroscopic data of **1** were similar to those of myrocin B (**8**); data for the related pimarane diterpene were published earlier.[3] The main difference between **1** and myrocin B was the replacement of a hydroxymethine group with a carbonyl group at C7 and an oxymethine carbon with an oxygenated quaternary carbon at C6. Needle-like crystals of **1** were obtained, which allowed the structure to be determined by X-ray diffraction analysis. The absolute configurations of C1, C4, C5, C6, C7, C9, C10, and C13 were determined to be *S*, *S*, *R*, *S*, *R*, *S*, *S*, and *R*, respectively, which were deduced from the Flack parameter,[6] 0.0 (2), refined using 1313 Friedel pairs (Figure 1). Hence, the structure of **1** was determined at the level of its absolute configuration, and the compound was named myrocin D.

Compound **2** was obtained as colorless needle-like crystals, and the molecular formula was deduced to be $C_{20}H_{26}O_5$ by HREIMS. The ^{13}C NMR spectrum (Table 2) exhibited the presence of one carbonyl carbon signal at C-7 (δ_C 178.8) and three methyl carbon signals at C-20 (δ_C 17.2), C-17 (δ_C 23.7), and C-19 (δ_C 24.9). Furthermore, the ^1H NMR spectrum (Table 2) showed four olefinic protons at H-14 (δ_H 6.92), H-15 (δ_H 5.83), H-16a (δ_H 5.03), and H-16b (δ_H 5.06). The ^{13}C NMR data of **2** were similar to those of libertellenone C (**6**), except δ_C 107.6 in **2** replaced a primary alcohol in **6**. This carbon was assigned as C-18 on the basis of HMBC correlations of H-19 (δ_H 1.30) to C-18 (δ_C 107.6), and H-18 (δ_H 5.36) to C-4 (δ_C48.9), C-5 (δ_C 145.8), and C-19 (δ_C 24.9). The absolute configuration of **2** was confirmed by X-ray diffraction analysis (Figure 2), and the compound was named libertellenone E.

Compound **3** was obtained as colorless needle-like crystals, and the molecular formula was determined to be $C_{20}H_{26}O_4$ by HREIMS. The ^{13}C and ^1H NMR spectral data of **3** (Table 2) showed similarities to those of **2**, except the oxycarbon (δ_C 85.8) in **3** replaced the dioxycarbon (δ_C 107.6) in **2**. This oxycarbon was assigned as C-18 by the HMBC correlations of H-19 (δ_H 1.34) to C-18 (δ_C 85.8), H-18b (δ_H 3.91) to C-3 (δ_C 34.3), C-4 (δ_C42.3), and C-19 (δ_C 25.4), and H-18a (δ_H 4.20) to C-4, C-6 (δ_C 148.4), and C-19. The absolute configuration of **3** was confirmed by X-ray diffraction analysis (Figure 3), and the compound was named libertellenone F.

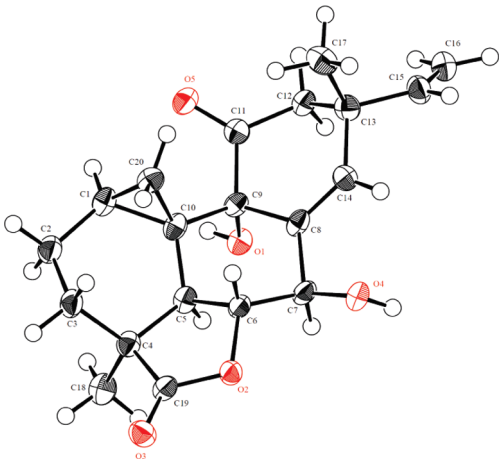

Figure 1. ORTEP drawing of **1** obtained by X-ray analysis [Flack parameter: x = 0.0(2)].

The molecular formula of compound **4** was determined to be $C_{12}H_{12}O_5$ based on HREIMS. The ^{13}C NMR spectrum (Table 3) showed one ester carbon signal at C-1 (δ_C 167.3), one hydroxyl methylene signal at C-11 (δ_C 59.9), and two methyl carbon signals at C-12 (δ_C 17.1) and C-13 (δ_C 14.2). Furthermore, the ^1H NMR spectrum (Table 3) showed one aromatic proton signal at H-7 (δ_H 6.89, 1H, s) and a hydrogen-bonded proton signal at 8-OH (δ_H 12.57, 1H, s). HMBC correlations were observed from H-13 to C-5, C-6, and C-10, H-12 to C-3, C-4, and C-10, H-11 to C-3 and C-4, H-7 to C-5, C-6, C-8, and C-9, and 8-OH to C-7 and C-8. These data allowed the structure of **4** to be deduced as an isocoumarin. The spectral data of **4** were similar to those of decarboxycitrinone.[7] The main difference between **4** and decarboxycitrinone was the replacement of a primary alcohol with a methyl C-11. Thus, the structure of **4** was established, and the compound was named decarboxyhydroxycitrinone. The structure of **4** was supported by X-ray diffraction analysis (Figure 4).

Two known diterpenes, myrocin A (**5**) and libertellenone C (**6**), and a known compound, cytochalasin E (**7**), were identified on the basis of their spectroscopic profiles (NMR, UV, IR, MS, $[\alpha]_D$) and comparison to published data.

Angiogenesis plays a critical role in the growth of tumor cells, and tumor angiogenesis inhibitors are considered novel targets for anticancer therapy.[8−10] Pathological angiogenesis is characterized by the persistent proliferation of endothelial cells and blood vessel formation.[11] Therefore, we studied the ability of isolated compounds **1**−**7** to inhibit the proliferation of HUVECs and HUAECs, using the MTT assay to evaluate antiangiogenesis (Table 4). Decarboxyhydroxycitrinone (**4**) and myrocin A (**5**) showed antiproliferative activity against HUVECs and HUAECs, and libertellenone C (**6**) showed weak antiproliferative activity against HUVECs and HUAECs. In contrast, cytochalasin E (**7**)

Table 2. NMR Spectroscopic Data (400 MHz, δ in ppm) for Compounds 2 and 3

	libertellenone E (**2**)[a]				libertellenone F (**3**)[b]			
position	δ_C		δ_H (J in Hz)	HMBC	δ_C		δ_H (J in Hz)	HMBC
1	69.1	CH	4.36, m		70.1	CH	4.43, dd (11.5, 4.4)	
2α	28.0	CH_2	1.83, m		29.7	CH_2	1.77, m	1, 4
2β			1.90, m	4			1.89, m	1, 4
3α	26.4	CH_2	1.47, dd (13.2, 2.5)		34.3	CH_2	1.79, m	1, 5
3β							1.59, dd (13.8, 3.8)	2
4	48.9	qC			46.3	qC		
5	145.8	qC			148.8	qC		
6	144.5	qC			148.4	qC		
7	178.8	qC			180.2	qC		
8	136.2	qC			138.3	qC		
9	76.1	qC			76.0	qC		
10	47.3	qC			48.5	qC		
11α	28.1	CH_2	1.93, m	8, 9, 13	29.3	CH_2	2.04, ddd (15.1, 15.1, 3.9)	
11β			2.21, m				2.29, m	
12α	29.8	CH_2	1.76, m	9, 13	31.2	CH_2	1.84, m	11, 17
12β			1.76, m	9, 13			1.52, m	
13	38.3	qC			39.3	qC		
14	147.7	CH	6.92, s	7, 8, 9, 12, 13, 15, 17	147.2	CH	6.85, d (1.2)	7, 8, 9, 12, 13, 15
15	145.5	CH	5.83, dd (17.2, 10.6)	12, 13, 14, 17	147.2	CH	5.90, dd (17.5, 10.6)	12, 13, 14, 17
16a	112.6	CH_2	5.03, d (10.6)	13, 15	112.6	CH_2	5.04, d (10.6)	13, 15
16b			5.06, d (17.2)	13, 15			5.09, d (17.5)	13, 15
17	23.7	CH_3	1.11, s	12, 13, 14, 15	24.0	CH_3	1.14, s	12, 13, 14, 15
18a	107.6	CH	5.36, s	4, 5, 19	85.8	CH_2	4.20, d (8.3)	4, 6, 19
18b							3.91, d (8.3)	3, 4, 19
19	24.9	CH_3	1.30, s	3, 4, 5, 18	25.4	CH_3	1.34, s	3, 4, 5, 18
20	17.2	CH_3	1.19, s	1, 5, 9, 10	18.0	CH_3	1.20, s	1, 5, 9, 10

[a] In $CDCl_3$. [b] In CD_3OD.

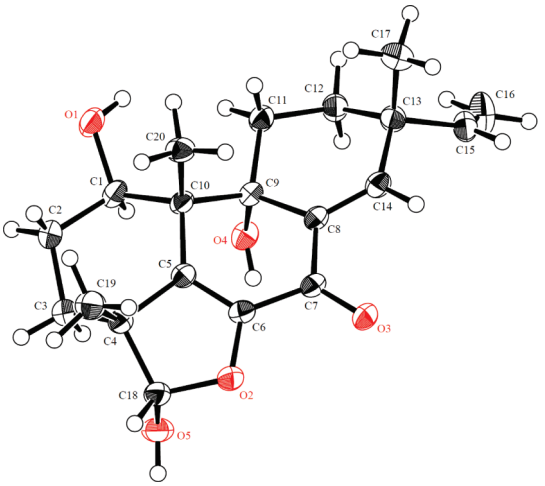

Figure 2. ORTEP drawing of **2** obtained by X-ray analysis [Flack parameter: x = 0.02(16)].

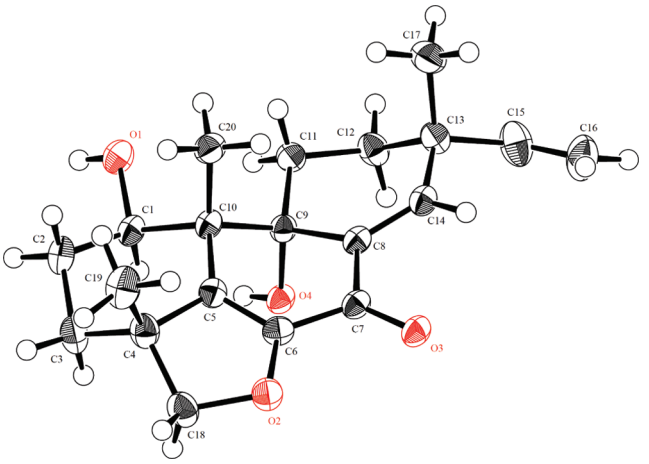

Figure 3. ORTEP drawing of **3** obtained by X-ray analysis [Flack parameter: x = 0.08(17)].

markedly inhibited proliferation of HUVECs and HUAECs. The activities of **7** were higher than those of Ki8751 (IC_{50} = 1.0−2.0 μM),[12] used as a positive control. The antiangiogenic activity of cytochalasin E (**7**) has already been published by T. Udagawa et al.[13]

EXPERIMENTAL SECTION

General Experimental Procedures. Melting points were determined on a Yanaco MP apparatus. Optical rotation was measured with a Horiba SEPA-300 polarimeter. IR spectra were recorded with a Jasco IR Report-100 spectrophotometer. ^1H NMR and ^{13}C NMR spectra were

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Table 3. NMR Spectroscopic Data (400 MHz, C_5D_5N, δ in ppm) for Decarboxyhydroxycitrinone (4)

position	δ_C		δ_H (J in Hz)	HMBC
1	167.3	qC		
2				
3	152.3	qC		
4	112.6	qC		
5	113.2	qC		
6	165.8	qC		
7	102.4	CH	6.89, s	5, 6, 8, 9
8	162.8	qC		
8-OH			12.57, s	7, 8
9	100.6	qC		
10	139.5	qC		
11α,β	59.9	CH_2	4.74, s	3, 4
12	17.1	CH_3	2.43, s	3, 4, 10
13	14.2	CH_3	2.55, s	5, 6, 10

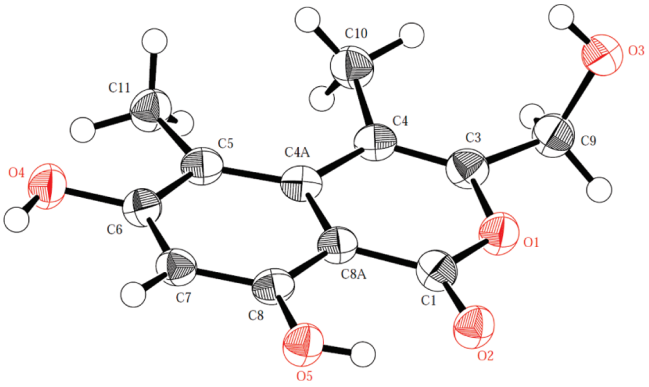

Figure 4. ORTEP drawing of **4** obtained by X-ray analysis.

measured with a Jeol JNM-AL400 MHz spectrometer using tetramethylsilane as the internal standard. Low- and high-resolution EIMS spectra were measured with a Jeol JMS-700 spectrometer. Column chromatography was performed using silica gel 60N (63-210 μm) from Kanto Chemical and Sephadex LH-20 from GE Healthcare. X-ray analysis was conducted using a Rigaku RAXIS RAPID imaging plate area detector with graphite-monochromated Cu Kα radiation.

Fungal Material. The fungus was isolated from the surface of an unidentified sponge collected from the coast of Atami-shi, Shizuoka Prefecture, Japan, in April 2006. The isolate was identified by rDNA sequence analysis. The internal transcribed spacer regions 1 and 2 and 5.8S rDNA in the rRNA gene of the isolate were identical to those of an epitype strain of *Arthrinium sacchari* (accession number: AF393679). The DNA sequence data have been deposited in the DDBJ as AB538274.

Fermentation. *A. sacchari* was inoculated into 500 mL Roux flasks (20 flasks) containing wheat (150 g per flask) and artificial seawater (50 mL per flask). The flasks were incubated at 25 °C in the dark for 14 days.

Extraction and Isolation. The fermented wheat substrate was extracted with $CHCl_3$ and EtOAc. The $CHCl_3$ extract (28.19 g) was fractionated by silica gel column chromatography with $CHCl_3$−MeOH (100:1, 50:1, 20:1, 10:1, 5:1, 0:100) to yield fractions A−H. Fraction C (1.02 g) was then subjected to three chromatography steps: (1) step-gradient silica gel column chromatography with $CHCl_3$−MeOH (100:1, 50:1, 30:1, 0:100); (2) Sephadex LH-20 column chromatography with MeOH; and (3) step-gradient silica gel column chromatography with *n*-hexane−acetone (15:1, 10:1, 7:1, 5:1, 3:1) followed by MeOH to yield 10 fractions; cytochalasin E (**7**) (97.5 mg) was isolated from fraction 8, while myrocin D (**1**) (7.3 mg) was precipitated with $CHCl_3$ from fraction 5. Fraction D (1.10 g) was then subjected to three chromatography steps: (1) step-gradient silica gel column chromatography with $CHCl_3$−MeOH (100:1, 50:1, 30:1, 0:100); (2) step-gradient silica gel column chromatography with *n*-hexane−acetone (5:1, 3:1, 1:1, 0:100) followed by MeOH; and (3) Sephadex LH-20 column chromatography with MeOH to yield four fractions; libertellenone F (**3**) (19.5 mg) was isolated from fraction 2. The EtOAc extract (16.55 g) was subjected to silica gel column chromatography with $CHCl_3$−MeOH (100:1, 50:1, 20:1, 10:1, 5:1, 0:100) to yield fractions A−H. Fraction E was then subjected to two chromatography steps: (1) step-gradient silica gel column chromatography with $CHCl_3$−MeOH (100:1, 50:1, 30:1, 0:100); and (2) Sephadex LH-20 column chromatography with MeOH to yield seven fractions; myrocin A (**5**) (15.1 mg) was isolated from fraction 3, while decarboxyhydroxycitrinone (**4**) (7.5 mg) was precipitated with MeOH from fraction 5. Fraction F was then subjected to three chromatography steps: (1) step-gradient silica gel column chromatography with $CHCl_3$−MeOH (100:1, 50:1, 20:1, 10:1, 5:1, 0:100); (2) Sephadex LH-20 column chromatography with MeOH; and (3) step-gradient silica gel column chromatography with *n*-hexane−acetone (1:1, 1:3, 0:100) followed by MeOH to yield four fractions; libertellenone C (**6**) (4.2 mg) was isolated from fraction 1, while libertellenone E (**2**) (21.6 mg) was isolated from fraction 2.

Table 4. Growth Inhibition of Compounds 1−7 against HUVECs and HUAECs

	IC_{50} (μM)	
	HUVECs	HUAECs
1	70.8	277.1
2	236.4	237.7
3	64.1	97.8
4	7.6	17.4
5	11.0	9.2
6	29.7	68.7
7	0.0114	0.0110

Myrocin D (1): colorless powder; mp 218−221 °C; $[\alpha]^{21}_D$ −298 (c 0.29, MeOH); IR (KBr) ν_{max} 3400, 2982, 1740, 1715, 1130 cm^{-1}; ^{13}C NMR (CD_3OD) see Table 1; ^1H NMR (CD_3OD) see Table 1; EIMS m/z 344 $[M]^+$ (15), 326 (100), 298 (39), 276 (38), 269 (44), 247 (25), 230 (30), 181 (55), 135 (34), 105 (31), 93 (35), 91 (51), 79 (35); HREIMS m/z 344.1621 $[M]^+$ (calcd for $C_{20}H_{24}O_5$, 344.1624).

X-ray Crystallographic Data of 1. Myrocin D (**1**) was crystallized from *n*-hexane−$CHCl_3$−MeOH to give colorless needles. Crystal data: $C_{20}H_{24}O_5$, space group $P2_1$ (#4), a = 10.5854(7) Å, b = 7.81311(15) Å, c = 10.6146(8) Å, β = 98.851(7)°, V = 867.43(9) $Å^3$, Z = 2, D_{calc} = 1.319 g/cm^3, R = 0.0748, wR_2 = 0.1374. The absolute configuration was determined on the basis of a Flack parameter of 0.0(2),[6] refined using 1313 Friedel pairs. Crystallographic data for **1** reported in this paper have been deposited at the Cambridge Crystallographic Data Centre, under reference number CCDC 758154. The data can be obtained free of charge at http://www.ccdc.cam.ac.uk/cgi-bin/catreq.cgi, or from the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK, fax: +44-1223-336-033; e-mail: data_request@ccdc.cam.ac.uk.

Libertellenone E (2): colorless needles; mp 234−237 °C; $[\alpha]^{19}_D$ +21 (c 0.21, CH_3OH); UV (CH_3OH) λ_{max} (log ε) 313 (3.87), 268 (3.64), 205 (3.77) nm; IR (KBr) ν_{max} 3425, 1650, 1635, 1540, 1457, 1068 cm^{-1}; ^{13}C NMR ($CDCl_3$) see Table 2; ^1H NMR ($CDCl_3$) see Table 2; EIMS m/z 346 $[M]^+$ (100), 328 (93), 313 (45), 165 (75); HREIMS m/z 346.1783 $[M]^+$ (calcd for $C_{20}H_{26}O_5$, 346.1780).

X-ray Crystallographic Data of 2. Libertellenone E (**2**) was crystallized from *n*-hexane−$CHCl_3$ to give colorless needles. Crystal data: $C_{20}H_{26}O_5$, space group $P2_12_12_1$ (#19), a = 11.9928(3) Å, b = 12.0355(3) Å, c = 12.1352(9) Å, V = 1751.6(2) Å^3, Z = 4, D_{calc} = 1.314 g/cm^3, R = 0.0429, wR_2 = 0.1015. The absolute configuration was determined on the basis of a Flack parameter of 0.02(16), refined using 1366 Friedel pairs. Crystallographic data for **2** reported in this paper have been deposited at the Cambridge Crystallographic Data Centre, under reference number CCDC 807680. The data can be obtained free of charge.

Libertellenone F (3): colorless needles; mp 233−236 °C; $[\alpha]^{19}_D$ −40 (c 0.29, CH_3OH); UV (CH_3OH) λ_{max} (log ε) 321 (3.85), 264 (3.63), 205 (3.81) nm; IR (KBr) ν_{max} 3448, 1681, 1652, 1638, 1558, 1077 cm^{-1}; ^{13}C NMR (CD_3OD) see Table 2; ^{1}H NMR (CD_3OD) see Table 2; EIMS m/z 330 $[M]^+$ (4), 312 (96), 255 (100), 175 (11); HREIMS m/z 330.1829 $[M]^+$ (calcd for $C_{20}H_{26}O_4$, 330.1831).

X-ray Crystallographic Data of 3. Libertellenone F (**3**) was crystallized from $CHCl_3$−MeOH to give colorless needles. Crystal data: $C_{20}H_{26}O_4$, space group $P2_12_12_1$ (#19), a = 11.3885(3) Å, b = 11.8068 (5) Å, c = 12.5872(9) Å, V = 1692.5(2) Å^3, Z = 4, D_{calc} = 1.297 g/cm^3, R = 0.0421, wR_2 = 0.1015. The absolute configuration was determined on the basis of a Flack parameter of 0.08(17), refined using 1309 Friedel pairs. Crystallographic data for **3** reported in this paper have been deposited at the Cambridge Crystallographic Data Centre, under reference number CCDC 807681. The data can be obtained free of charge.

Decarboxyhydroxycitrinone (4): colorless needles; mp 253−256 °C; UV (DMSO) λ_{max} (log ε) 336 (3.61), 268 (3.86) nm; IR (KBr) ν_{max} 3999, 2920, 1650, 1558, 1539, 1090 cm^{-1}; ^{13}C NMR (C_5D_5N) see Table 3; ^{1}H NMR (C_5D_5N) see Table 3; EIMS m/z 236$[M]^+$ (100), 207 (28), 177 (56), 149 (26); HREIMS m/z 236.0689 $[M]^+$ (calcd for $C_{12}H_{12}O_5$, 236.0685).

X-ray Crystallographic Data of 4. Decarboxyhydroxycitrinone (**4**) was crystallized from $CHCl_3$−MeOH to give colorless needles. Crystal data: $C_{12}H_{12}O_5$, space group $P2_1/c$ (#14), a = 9.6413(7) Å, b = 15.5234(3) Å, c = 6.86279(12) Å, V = 995.48(7) Å^3, Z = 4, D_{calc} = 1.576 g/cm^3, R = 0.0707, wR_2 = 0.1600. Crystallographic data for **4** reported in this paper have been deposited at the Cambridge Crystallographic Data Centre, under reference number CCDC 826342. The data can be obtained free of charge.

Cell Culture. HUVECs and HUAECs were purchased from Lonza Walkersville, Inc. HUVECs were cultured using an EGM-2 Bulletkit (Lonza Walkersville, Inc.) at 37 °C in 5% CO_2. HUAECs were cultured using an EGM-2MV Bulletkit (Lonza Walkersville, Inc.) at 37 °C in 5% CO_2.

Growth Inhibition Assay. Cells (3×10^3 cells/well) were seeded in 96-well plates with culture medium for 3 h at 37 °C in 5% CO_2. The medium was removed and replaced with 1% FBS−EBM-2 and incubated for 21 h at 37 °C in 5% CO_2. VEGF (1 nM) and the test compounds were added to each well and incubated for 72 h at 37 °C in 5% CO_2. Cell proliferation was detected using WST-8 reagent, and the inhibition of proliferation was measured at an absorbance wavelength of 450 nm using a plate reader. Ki8751 (Calbiochem), which is a VEGFR2 kinase inhibitor, was used as a positive control.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. ^{1}H and ^{13}C NMR spectra of **1**−**4** are available free of charge via the Internet at http://pubs.acs.org.

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